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Full Length Article

Molecular Characterization, Structure and Expression Analysis of a Ras-Related Nuclear (*Ran*) Gene in Pacific White Shrimp, *Litopenaeus vannamei*

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Abstract

Ras-related nuclear (*Ran*) gene is widely expressed and predominantly localized in the nucleus of eukaryotic cells. It has been shown to function in nucleo-cytoplasmic transport, mitotic spindle formation and nuclear envelope reformation. Pacific white shrimp (*Litopenaeus vannamei*, Boone, 1931) is an economic culture species all over the world. With the rapid development of shrimp cultures, bacterial and viral diseases become serious problems in its aquaculture. However, little is known about the role of *Ran* gene in *L. vannamei* against viral or bacterial infection. The present study showed that full-length of *L. vannamei Ran* cDNA was 1104 bp, including a 5'-terminal untranslated region (UTR; 71 bp), 3'-UTR (387 bp) and 645 bp of open reading frame encoding 215 amino acids. *Ran* gene was well conserved among shrimp, bee and ant species. This protein contains an ATP/GTP binding domain and an effector molecule binding motif. Like human *Ran*, *L. vannamei Ran* was consisted of multiple α-helix and β-sheets structures, and ATP/GTP binding motifs were located on the surface of structure. Quantitative RT-PCR analysis demonstrated that *Ran* was expressed highly in lymphoid organs and gills, followed in hepatopancreas and hemocytes. Time-course expression analysis indicated that *Ran* transcript was up-regulated in groups infected with both *Vibrio anguillarum* and white spot syndrome virus (WSSV). It suggests that *Ran* may play roles in *L. vannamei* immunity against both viral and bacterial infections, which provided valuable information for further studying the functions and regulation mechanism of *Ran* in *L. vannamei.* © 2019 Friends Science Publishers

Keywords: Immune function; *Litopenaeus vannamei*; Molecular characterization; *Ran* gene structure

Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is native to Pacific coast of Latin America and it was introduced to coastal Asian countries in the early 1990s (Briggs *et al*., 2004). *L. vannamei* was rapidly developed into a primarily economic culture species in Asian countries and all over the world. However, with the rapid development of shrimp culture, bacterial and viral diseases occurred frequently and caused great economic losses. These diseases caused by bacteria or virus, such as *Vibrio anguillarum* and white spot syndrome virus (WSSV), limited its sustainable culture industry development (Bachere, 2000; Luo *et al.*, 2007). Therefore, understanding immune response of *L. vannamei* against viral or bacterial diseases has become an urgent mission for developing disease control and prevention and long-term sustainability.

Ras-related nuclear (*Ran*) gene belonged to subfamilies of small GTPases, is well conserved in eukaryotes from yeast to humans (Han *et al.*, 2010). *Ran* alternates between its GDP- and GTP-bound states, and functions mainly in nuclear transport and spindle assembly (Han *et al.*, 2010). The *Ran* gene of kuruma shrimp (*Marsupenaeus japonicus*) was cloned, and the expression was up-regulated in both WSSV-resistant and -infected shrimp at 4 h post infection (hpi), which indicated that *Ran* gene may play a role in the antiviral immune response of shrimp by enhancing hemocytic phagocytosis through interaction with myosin (Han and Zhang, 2007; Liu *et al.*, 2009). However, there is limited information about the structure of *Ran* gene in the widely cultured *L. vannamei* and its immune function. The present study aimed to clone *Ran* gene from *L. vannamei*, analyze the structure and determine immune function against both viral and bacterial infection. In practice, multiple infection was often detected

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in *L. vannamei* culture (Jang *et al*., 2015). Thus, the mRNA expression of *Ran* gene in *L. vannamei* infected by more pathogens was also examined to provide basic information to further illustrate the mechanism why multiple infections caused more mortality than single infection (Jang *et al*., 2015).

Materials and Methods

Shrimp

L. vannamei (12 ± 1.4 g, mean ± SD) were obtained from the Taean center at West Sea Research Institute, NFRDI, Korea. Prior to the experiments, shrimp were acclimated in a static aquarium in seawater with 30 practical salinity units (psu) and pH 7.7–8.3 at 27–28ºC under a 12D/12L cycle for two weeks. Shrimp were fed with commercial diets (CJ Feeding Company, Korea) at 3% of their body weight four times daily. During the entire experimental period, 40–50% of water was exchanged daily, and dissolved oxygen (DO) levels were kept more than 5 mg/L.

RNA Isolation

Whole bodies of shrimp were washed in sterilized seawater, slightly dried with Kimtech wipes, cut into small pieces, and mixed on ice. Then, the pieces were immediately placed into 200 μL of RNA-later reagents (Ambion, Austin, TX, USA). Total RNA was extracted (RNeasy Mini kit, Qiagen, Valencia, CA, USA), purified with DNase I (Qiagen) according to the manufacturer's protocol, and stored at -80°C until cDNA synthesis.

Rapid Amplification of cDNA Ends (RACE)

The 3'-RACE and 5'-RACE-ready cDNA were synthesized separately with the SMARTer™ RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. The 3'-RACE-ready cDNA was extended with a Poly(A) tail using Poly(A) polymerase (Takara Bio, USA). These cDNA were diluted 20 times and used as RACE templates. Degenerate gene-specific primers called *Ran*-3'-RACE (GSP1, Table 1) and *Ran*-5'-RACE (GSP2, Table 1) were designed based on *Ran* sequences from *M. japonicus*. PCR amplification was conducted with primers (GSP1 primer and universal UPM primer for 3'-RACE, GSP2 and UPM for 5'-RACE) provided by the kit. PCR conditions were as follows: five cycles of 94°C for 30 s and 72°C for 3 min, followed by five cycles of 94°C for 30 s, 70°C for 30 s, and 72°C for 3 min, then 25 cycles of 94°C for 30 s, 67°C for 3'-RACE (or 66°C for 5'-RACE) for 30 s, and finally 72°C for 3 min. PCR products were checked on a 1.2% agarose gel, cloned into TOPO vector (Invitrogen, Carlsbad, CA, USA) and sequenced. The fragment sequence was confirmed with BLAST program on National Center for Biotechnology Information (NCBI) website. After 3'-RACE sequence and 5'-RACE sequence was confirmed through BLAST analysis, their fragment sequences were joined with BioEdit 7.0.5 package (Wang *et al*., 2007).

Sequence Analysis

The full sequence of *L. vannamei Ran* gene was deposited into GenBank (NCBI) database and accession number was JX644455. The protein motifs and open reading frame (ORF) of gene were analyzed with Expasy search program (http://au.expasy.org/tools) and ORF finder program on NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), respectively. The molecular weight (MW) and theoretical isoelectric point (pI) of proteins were estimated online (http://isoelectric.ovh.org). SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP) was used to predict signal peptide. BLASTN and BLASTP search programs in NCBI were performed to analyze nucleotide sequence and deduced amino acid sequences of *Ran*, respectively. Multiple alignments were performed with CLUSTAL-W program in BioEdit 7.0.5 package (Wang *et al*., 2007). A neighbor-joining phylogenetic tree based on the deduced amino acid sequences was constructed with MEGA 5 program (Tamura *et al*., 2007). The resulting tree topologies were evaluated according to p-distance model with neighbor joining method, and bootstrap analysis was conducted with 1000 replications (Saitou and Nei, 1987). The 3-D structure of *Ran* was constructed by comparative model method with Swiss-Model software and structure quantity was checked by PROCEHCK software.

Tissue-specific Distribution

Tissues including muscle, antenna gland, stomach, gill, nerve cord, hepatopancreas, heart, intestine and gill were dissected from *L. vannamei*. Hemocytes and hemolymph were sampled following description by Jang *et al.* (2011). All tissues and pellets were diluted using 200 μL of RNA later reagents. According to the method of Qiao *et al.* (2015), total RNA extraction, cDNA synthesis and mRNA transcription level were performed. Then, RT-qPCR was conducted with the One Step PrimeScript™ RT-PCR perfect real time kit (Takara Bio., Japan). The final 20 μL reaction volume was used, including 10 μL of 2× RT-PCR Buffer III, 0.4 μL of reverse transcript enzyme Mix II, 2 units of Ex Hot Start Taq enzyme, 0.4 μM of each forward/reverse primers and Taqman probe (Table 1). The RT-qPCR reaction conditions were as follows: 42°C for 5 min, 95°C for 10 s, then 40 cycles of 95°C for 5 s and 60°C for 30 s. Initiation of fluorescent signal at annealing stage during the first cycle was detected. β-actin as an internal reference was amplified with the primers and probe (Table 1). All samples were analyzed in triplicate, and comparative threshold cycle (CT) method ($2^{-\Delta\Delta CT}$ method) was used to analyze the relative expression of target gene (Livak and Schmittgen, 2001).

Table 1: Primers and probes used for *Ran* in this study

Name	Nucleotide sequences (5'-3')	Note
GSP1	TCCCTATTGTACTCTGCGGAACCAAGGT	3' RACE primer
GSP2	GGAGGCTTCCTGGAGGTCGTTCT	5' RACE primer
q-*Ran*-F	CCAAGAGAAATTGGGAGGTCTTC	RT-qPCR forward primer
q-*Ran*-R	GGGAACATTCTTGTACGTGACTCTAG	RT-qPCR reverse primer
q-*Ran*-P	ATGGTTACTACATCCAGGCCCACTGTGC	RT-qPCR probe
q-β-actin-F	CGAGGTATCCTCACCCTGAAAT	RT-qPCR forward primer
q-β-actin-R	GTGATGCCAGATCTTCTCCATGT	RT-qPCR reverse primer
q-β-actin-P	CGAGCACGGCATCGTCACCAA	RT-qPCR probe

Immune Challenge by Virus and Bacteria

Experimental shrimp: Normal *L. vannamei* (1.2 ± 0.2 g) were obtained from the center and maintained in the same conditions as described in section 2.1.

Bacterial suspension: Prior to challenge tests, *V. anguillarum* KCTC 2711 was cultured three times in tryptic soy agar with final 2% NaCl for 24 h at 27ºC. The colonies were collected and washed with sterilized physiological saline (PS) by centrifugation. Then, the final bacterial suspension at concentrations of 3.3×10^6 and 3.3×10^3 CFU/mL was prepared with PS according to $OD_{595\ nm}$ detection.

Viral stock: The WSSV stocking solution was prepared following the method of Yun *et al.* (2014), and then was diluted with TNE buffer (50 m*M* Tris-HCl, 5 m*M* EDTA, 400 m*M* NaCl, 1 m*M* proteinase inhibitor PMSF, pH8.5) containing 1.5% 4-nonylphenyl-polyethylene glycol (NP40, Sigma) to obtain working solution for further challenge tests.

Challenge tests with virus or bacteria: Challenge tests including single infection and multiple infections, were conducted following the previous description by Jang *et al.* (2015), Qiao *et al. (*2015). In single challenge groups, shrimp were injected with 20 μL of WSSV suspension (2.0×10^2 copies/μL) or *V. anguillarum* (3.3×10^6 CFU/mL), and shrimp was injected with an equal volume of PS as control group. In super-infection group, shrimp were first injected with 20 μL of WSSV suspension (2.0×10^2 copies/μL), and then the WSSV-infected shrimp were again injected with *V. anguillarum* suspension (3.3×10^3 CFU/mL) at 24 h post injection (hpi). In co-infection groups, shrimp were simultaneously injected with 20 μL of cocktail mixed with WSSV (2.0×10^2 copies/μL) and *V. anguillarum* (3.3×10^3 CFU/mL). All the shrimp were injected in ventral sinus between the 4th and 5th pleopods via 0.3 mL insulin syringe (BD medical-diabetes care, USA). Seventy individuals were included in each group, and all the challenge tests were conducted in triplicate. Five individuals were sampled at 0, 3, 6, 12, 24, 48, 72 and 168 hpi from each group, and samples of whole body were used for time-course mRNA expression analysis.

Temporal mRNA expression in response to viral and bacterial infections: Total RNA isolation, cDNA synthesis and mRNA relative expression was performed as described in sections 2.2 and 2.5. All assays were performed in triplicate, and significant differences were compared to the control group (at time = 0 h).

Statistical Analysis

The experimental results were expressed as mean ± standard deviation (SD), and data were subjected to one-way analysis of variance using the statistical software program SPSS version 17.0 (SPSS Inc., IL, USA). Dunnett's *t*-test and Tukey's range test were used as post hoc test to compare means at values of $p < 0.05$ or less.

Results

Characterization of Full-length cDNA of *Ran* Gene from *L. vannamei*

The cDNA full-length of *Ran* gene was 1104 bp, including a 5'-terminal untranslated region (UTR) of 71 bp, 3'-UTR of 387 bp, and an ORF of 645 bp, which encoded a polypeptide of 215 amino acids (Fig. 1). The 3'-UTR included a stop codon (TAA), and a putative polyadenylation signals (AATAAA) located 33 bp upstream of poly (A) tail (Fig. 1). Multiple alignments based on the deduced amino acid sequence of ORF showed that this gene contained four conserved ATP/GTP binding motifs, which were well conserved in shrimp and other closely-related species (Fig. 2). The deduced protein of *Ran* contained an ATP/GTP binding domain and an effector molecule binding motif (Fig. 2). No signal peptide and transmembrane domain was found in *Ran* gene. Theoretical pI and MW were 7.33 and 24.63 kDa, respectively.

Phylogenetic Analysis of *Ran*

The phylogenetic tree and accession numbers are shown in Fig. 3. The *Ran* sequence of *L. vannamei* showed homology with that of other species. It showed 100, 99, 99, 92, 92, 92 and 92% identity with *Fenneropenaeus chinensis* [GenBank accession no. AFW98987], *Penaeus monodon* [AEO23963], *M. japonicus* [AAY96645], *Harpegnathos saltator* (Jerdon's jumping ant) [EFN84506], *Camponotus floridanus* (Tortugas carpenter ant) [EFN74765], *Apis mellifera* (European honey bee) [XP393761], and *Megachile rotundata* (Alfalfa leaf-cutting bee)

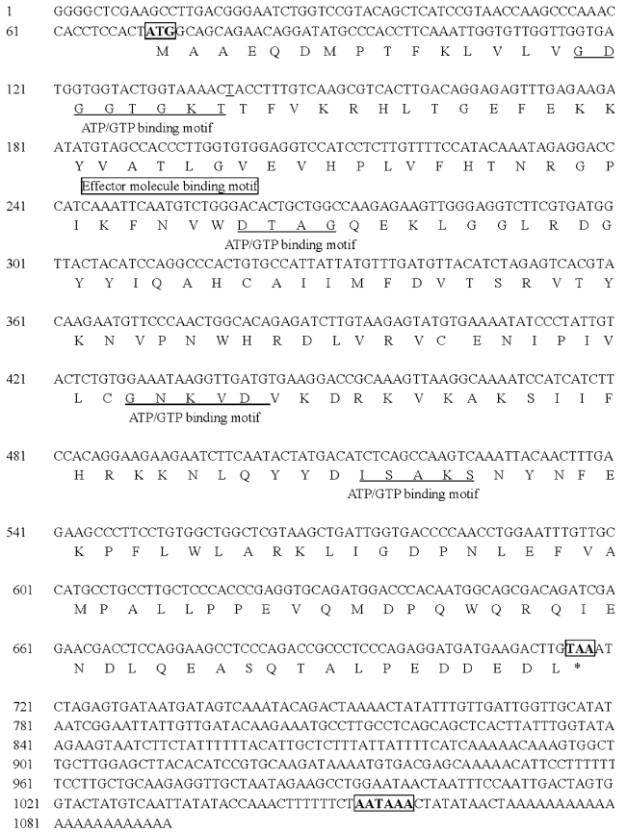

Fig. 1: Nucleotide and predicted amino acid sequence of a *ras*-related nuclear (*Ran*) gene from Pacific white shrimp (*Litopenaeus vannamei*). The bold letters in shaded boxes indicate the start codon (ATG), stop codon (TAA), and polyadenylation signal sequence (AATAAA). The ATP/GTP binding motif (underlined) is highly conserved in shrimp. The effector molecule binding motif is marked with gray shading

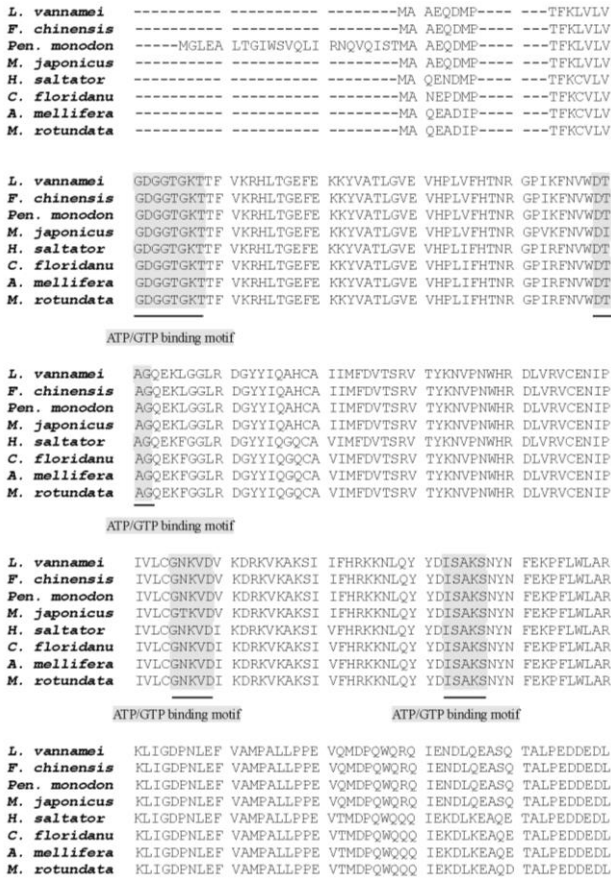

Fig. 2: Multiple amino acid sequence alignment of *Ran* protein in *L. vannamei* and some closely-related species. Gray shading indicates the ATP/GTP binding motif. GenBank accession numbers in NCBI are as follows: *P. monodon*, *Penaeus monodon* [AEO23963]; *M. japonicus*, *Marsupenaeus japonicus* [AAY96645]; *H. saltator*, *Harpegnathos saltator* [EFN84506]; *F. chinensis*, *Fenneropenaeus chinensis* [AFW98987]; *C. floridanus*, *Camponotus floridanus* [EFN74765]; *A. mellifera*, *Apis mellifera* [XP393761]; *L. vannamei*, *Litopenaeus vannamei*; and *M. rotundata*, *Megachile rotundata* [XP003699274]

[XP003699274], respectively.

3-D Structure Analysis of *Ran*

By searching the PDB database, human *Ran* (PDB No. 1QBK) which shared 88.37% identity with *L. vannamei Ran* (Fig. 4), was identified and chosen as template for 3-D construction. Results showed that *L. vannamei Ran* was consisted of four α-helix and seven β-sheets. The α-helix and seven β-sheets formed a groove, and the potential amino acids involved in GTP binding were located on the surface of groove (Fig. 5).

Tissue-specific Gene Expression in Normal Shrimp

Results of tissue-specific transcripts of *Ran* gene showed that *Ran* was expressed in each tissue examined *i.e.*, muscle, lymphoid organs, antenna, stomach, gill, nerve cord, hepatopancreas, heart, intestine and hemocytes (Fig. 6). The highest expression was found in lymphoid organs and gill ($p < 0.01$), followed in hepatopancreas and hemocytes (Fig. 6). The relative expression was similar in lymphoid organs and gill, and that level was about 1.95- and 4.36-folds higher than the expression levels in hepatopancreas and hemocytes, respectively.

Temporal mRNA Expression Responded to Bacterial and Viral Challenge

For a better understanding immune function of *Ran* gene against bacterial and viral pathogens infection, time-course mRNA expression was analyzed (Fig. 7).

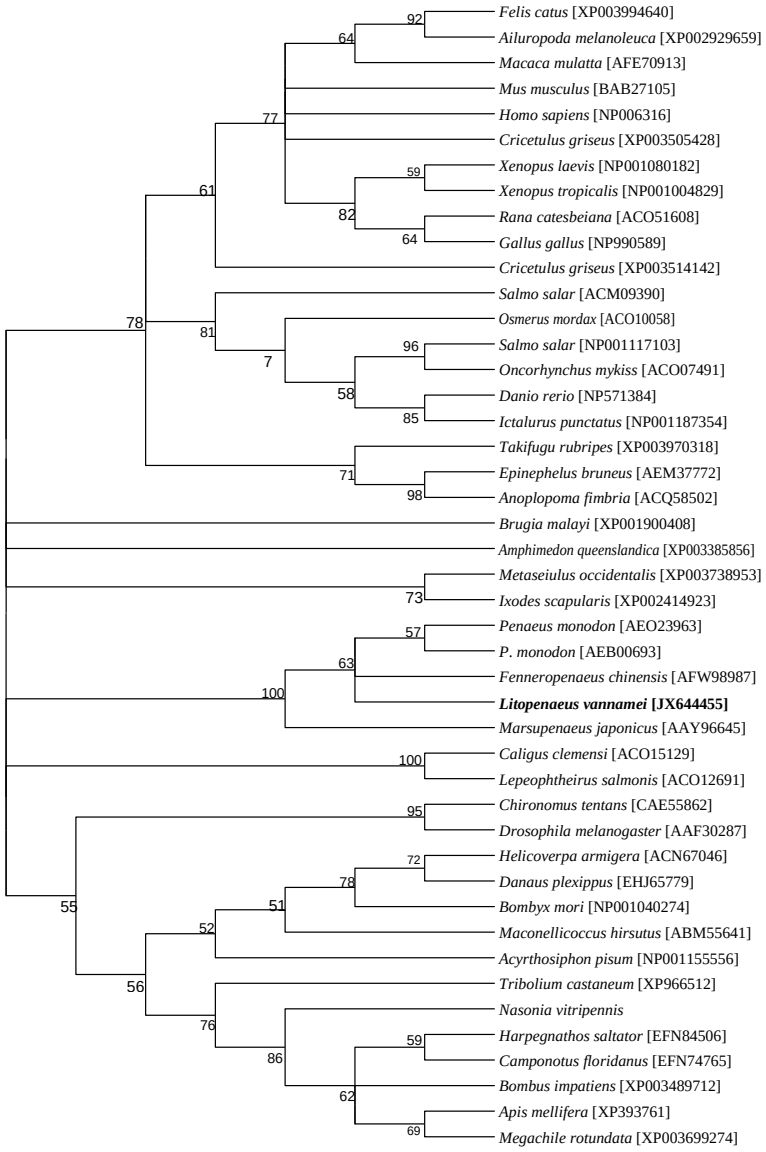

Fig. 3: Phylogenetic tree of species that express *Ran* sequences. The scale bar refers to a phylogenetic distance of 0.01 amino acid substitutions per site. The NCBI gene accession numbers are shown in brackets "[ ]". The *Ran* gene cloned in this study is marked in bold

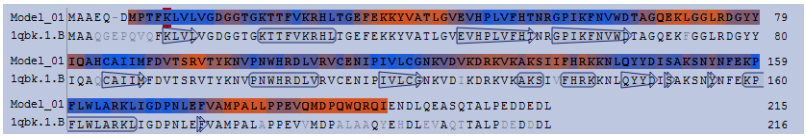

Fig. 4: Structural comparison of *L. vannamei Ran* and human *Ran* (PDB No. 1QBK)

In control group with PS injection, the relative expression of *Ran* was quickly up-regulated at 6 hpi, then began to decline, and the expression at 168 hpi were not different from that at 0 hpi. For *V. anguillarum* single-infection, the transcriptional levels were suppressed within a short time compared to that at 0 hpi, and up-regulated most by 9.1-folds at 12 hpi. Then, the transcription at 48 hpi began to decrease and returned to baseline level at 168 hpi. In WSSV single-infection group, the transcriptional levels were up-regulated by 1.9-folds at 6 hpi and decreased to baseline level after 24 hpi. However, the transcription was up-regulated by 5-folds again at 48 hpi and then declined again to be suppressed after 72 hpi. In co-infection group, transcriptional levels were decreased significantly until 48 hpi, and then began to recover to the baseline level. In super-infection group, expression levels decreased

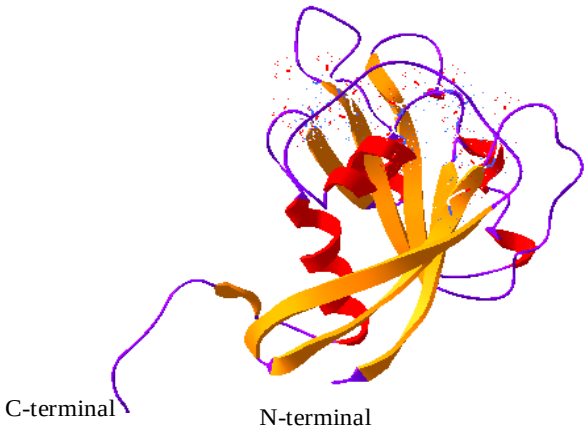

Fig. 5: 3-D structure of *L. vannamei Ran*. The amino acids involved in ATP/GTP binding are shown as color dots

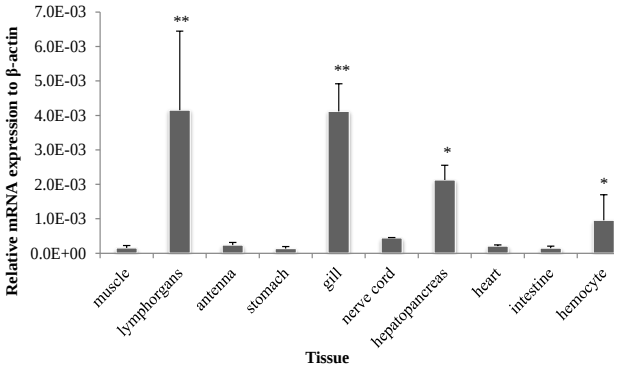

Fig. 6: Tissue-specific distribution of *Ran* gene in *L. vannamei*. The transcription level (Y-axis) was normalized to β-actin expression. Bars represent the mean ± SD of five replicates. Significant differences were determined with *Dunnett's t*-test and *Tukey's* range test by comparing to the expression in muscle, and are indicated with asterisks (**, $p < 0.01$; *, $0.01 < p < 0.05$)

significantly by 40% and then began to increase slightly. But it remained still lower than baseline level. However, transcriptional level was 2.4-folds higher than baseline level at 7 dpi.

Discussion

We identified and characterized the *Ran* gene from *L. vannamei*. The obtained cDNA of *L. vannamei Ran* was 1104 bp, encoding a polypeptide of 215 amino acids, which shared similar length with them of shrimp (*P. monodon* and *M. japonicus*), fish and human (Chook and Blobel, 1999; Seewald *et al*., 2002; Han *et al*., 2010, 2012). The sequence of *L. vannamei Ran* gene shared high identity with that of shrimp, ant, bee, frog and fish (Fig. 2 and 3). The structural analysis showed that *L. vannamei Ran* possessed similar 3-D structure to human *Ran* with ATP/GTP binding sites located on the surface of

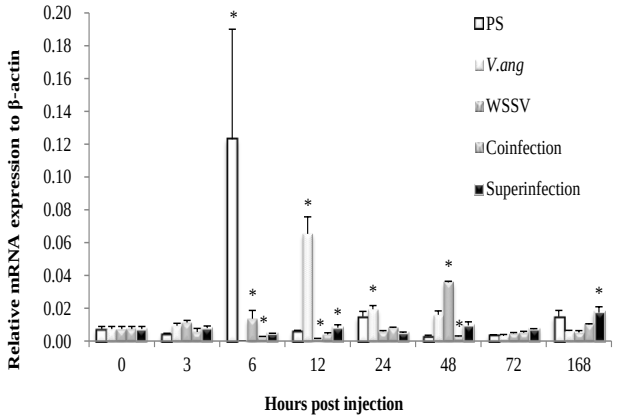

Fig. 7: Time-course mRNA expression of *Ran* in *L. vannamei* after infected with *Vibrio anguillarum*, WSSV, and both. Samples were measured at 0, 3, 6, 12, 24, 48, 72 and 168 hours post injection. Bars represent mean ± SD of five individual replicates at each time point for each group. Significant differences between each time point and baseline (time = 0 h) were determined with *Dunnett's t*-test and *Tukey's* range test. The significant levels are indicated by asterisks (*, $0.01 < p < 0.05$)

structure (Fig. 1). These results revealed that the function of *Ran* might be highly conserved in animals. *Ran* has been shown to alternate between GDP- and GTP-bound states, and it functions as an importnat regulator on nuclear transport and spindle assembly (Seewald *et al*., 2002). Theoretical MW of *L. vannamei Ran* was 24.63 kDa. This small size may benefit this protein for regulating nucleo-cytoplasmic transport. Dingwall and Laskey (1991) reported that molecules smaller than 20—40 kDa could passively diffuse through the nuclear pore complex (NPC). NPC possesses a large, complex and proteinaceous structure that provides a port into the nuclear envelope.

Ran gene was expressed constitutively in various tissues in the present study. Expression was highest in lymphoid organs and gill, followed in hepatopancreas and hemocytes. Although sequence of *L. vannamei Ran* shared high identity with other *Ran*, their expression pattern was slightly different. *M. japonicus Ran* was highly expressed in hepatopancreas (Han and Zhang, 2007) whilst *Ran* in large yellow croaker (*Larimichthys crocea*) was highly expressed in kidney (Han *et al*., 2010). The hepatopancreas and hemocytes are important immune-related tissues of shrimp species. High expression of *Ran* in these two tissues revealed that *Ran* might be involved in the immune reaction of *L. vannamei*. The different expression patterns of *Ran* in different animals indicated that *Ran* expression might be species-specific. It is well known that GTPases have many important roles in the regulation of biological processes, such as morphogenesis, growth and differentiation, cell division, cell motility and cytokinesis (Exton, 1998). Recently, *Ran* was proved to play important roles in fish immune response against

bacterial and viral infections, and *Ran* has been demonstrated to take part in shrimp immune response against viral infection (Han *et al*., 2010, 2012). The role of *Ran* in shrimp against bacterial infection is not very clear.

The present study further revealed that *Ran* is involved in the immune response of *L. vannamei* against both bacterial and viral infections. We found that stimulation with PS (control condition) induced rapid up-regulation of *Ran* gene at 6 hpi compared to that at 0 h. However, when *M. japonicus* and *L. crocea* were stimulated with PBS, no significant difference in *Ran* gene expression was observed at sampling time point during 2- or 3-day experiments (Han *et al*., 2010, 2012). We speculate that shrimp response to PS injection may have been a response to stress, in which living cells develop various molecular strategies naturally to defend themselves against various environmental stresses (Miyamoto *et al*., 2004). In fact, several gene expression patterns are known to be drastically altered in response to stress. Recently, it was reported that the collapse of a *Ran* gradient played an important role in the stress-induced nuclear accumulation of importing alpha (Miyamoto *et al*., 2004; Yasuda *et al*., 2006). Therefore, we hypothesize that an immediate up-regulation of *Ran* gene expression may have been a response to stimulation under control conditions (shrimp were stimulated with PS injection), and the expression level returned to baseline levels within a very short time. This might be considered a 'positive adaptive response' intended to protect against environmental stress. In the groups infected with either bacteria or virus, *Ran* mRNA expression was first depressed within a short time, then enhanced, and finally maintained at a level similar to that observed at 0 h. This suggested that *Ran* may play a role in shrimp immunity against both bacterial and viral infections. This was consistent with the results reported in *L. crocea*, where *Ran* gene expression was clearly up-regulated post-induction with viral mimetic, poly (I:C) and formalin-inactivated bacteria (Han *et al*., 2010). In addition, *Ran* gene expression was up-regulated when *M. japonicus* was infected with WSSV (Han *et al*., 2012). Our study demonstrated that *L. vannamei Ran* gene played roles in immune responses against both viral and bacterial infections.

WSSV is considered as an important pathoen in shrimp cultures. Meng *et al.* (2010) investigated WSSV infections in *L. vannamei* postlarvae at commercial farms in Korea. The results showed that most postlarvae were detected to be WSSV-positive with a low load, although all *L. vannamei* hatcheries were produced from SPF broodstocks in Hawaii (Jang *et al*., 2007). Previous studies showed that WSSV can often be detected in apparently healthy shrimp, although WSSV is highly virulent in shrimp (Manivannan *et al*., 2002; Natividad *et al*., 2006; Meng *et al*., 2010). The WSSV infection may result from the diverse biosecurity maintenance ways and feeds used in hatcheries, because it is thought that the infection can be attributed to water-borne or feed-borne virus. It was reported that rotifer (Yan *et al*., 2004; 2007) and bloodworm (Song *et al*., 2001) typically fed to broodstock and postlarvae shrimp, were WSSV-positive in some cases. Under field conditions, animals are often infected with more than one pathogen, including bacterial-bacterial, bacterial-viral or viral-viral multiple infections (Alapide-Tendencia and Dureza, 1997; Manivannan *et al*., 2002). Therefore, in this study, we conducted co- and super-infections with WSSV and bacteria to determine whether the immune response changed when WSSV-positive *L. vannamei* were again infected with bacteria. Our results demonstrated that *Ran* expression was significantly suppressed within 72 hpi, and then it gradually recovered in both co- and super-infection groups. This immune-suppression may be related to the increased mortality of shrimp under co- and super-infection conditions. For groups infected with *V. anguillarum* alone (10^6 CFU/mL), cumulative mortality was 12.5%. However, for groups co-infected and super-infected, cumulative mortality rates were 37.5 and 50% even at the lower bacterial concentration of 10^3 CFU/mL, respectively (Jang *et al*., 2015). Furthermore, shrimp in co- and super-infection groups died at earlier time points than shrimp in single-infection group. In addition, WSSV load in co- and super-infection groups increased more rapidly than that in single-infection group (Jang *et al*., 2015). These results suggested that a higher cumulative mortality and more rapid WSSV replication might be related to down-regulation of immune-related genes, like *Ran*. In accordance with that, the expression of proPO-activating factor (PPAF), proPO-activating enzyme 1 (PPAE1) and PPAE2 in the multiple-infection groups were suppressed more intensively than them in single-infection group (Qiao *et al*., 2015). Future studies should address immune pathway related to *Ran* gene expression.

Conclusion

Ran gene is well-conserved among different species. *Ran* gene may play role in *L. vannamei* immune response against both bacterial and viral diseases. Under multiple-infection conditions, the long period of suppressed *Ran* gene expression may facilitate pathogen invasion and replication.

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References

Alapide-Tendencia, E.V. and L.A. Dureza, 1997. Isolation of *Vibrio* spp. from *Penaeus monodon* (Fabricius) with red disease syndrome. *Aquaculture*, 154: 107–114

Briggs, M., S.F. Smith, R. Subasinghe and M. Phillips, 2004. *Introductions and movement of Penaeus vannamei and Penaeus stylirostris in Asia and Pacific*. FAO Regional Office for Asia and Pacific (RAP), RAP Publication, Bangkok, Thailand

Bachere, E., 2000. Shrimp immunity and disease control. *Aquaculture*, 191: 3–11

Chook, Y.M. and G. Blobel, 1999. Structure of the nuclear transport complex karyopherin-beta2-Ran x GppNHp. *Nature*, 399: 230–237

Dingwall, C. and R.A. Laskey, 1991. Nuclear targeting sequences - a consensus? *Trends Biochem. Sci*., 16: 478–481

Exton, J.H., 1998. Small GTPases minireview series. *J. Biol. Chem*., 273: 19923

Han, F. and X. Zhang, 2007. Characterization of a ras-related nuclear protein (Ran protein) up-regulated in shrimp antiviral immunity. *Fish Shellfish Immunol*., 23: 937–944

Han, F., X.Q. Wang, C.L. Yao and Z.Y. Wang, 2010. Molecular characterization of Ran gene up-regulated in large yellow croaker (*Pseudosciaena crocea*) immunity. *Fish Shellfish Immunol*., 29: 327–333

Han, F., X. Wang and Z. Wang, 2012. Molecular characterization of a Ran isoform gene up-regulated in shrimp immunity. *Gene*, 495: 65–71

Jang, I.K., J.C. Jun, G.J. Jo, Y.R. Cho, H.C. Seo, B.L. Kim and J.S. Kim, 2007. Polyculture of fleshy shrimp, *Fenneropenaeus chinensis* and Pacific white shrimp (*Litopenaeus vannamei*) with river puffer (*Takifugu obscurus*) in shrimp ponds. *J. Aquac*., 20: 278–288

Jang, I.K., Z.G. Pang, J.P. Yu, S.K. Kim, H.C. Seo and Y.R. Cho, 2011. Selectively enhanced expression of prophenoloxidase activating enzyme 1 (PPAE1) at a bacteria clearance site in the white shrimp (*Litopenaeus vannamei*). *BMC Immunol*., 12: 70–81

Jang, I.K., K. Qiao and S.K. Kim, 2015. Effect of multiple infections with white spot syndrome virus and *Vibrio anguillarum* on Pacific white shrimp (*Litopenaeus vannamei* L.): mortality and viral replication. *J. Fish Dis*., 37: 911–920

Liu, W., F. Han and X. Zhang, 2009. Ran GTPase regulates hemocytic phagocytosis of shrimp by interaction with myosin. *J. Proteome Res.*, 8: 1198–1206

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*, 25: 402–408

Luo, T., F. Li, K. Lei and X. Xu, 2007. Genomic organization, promoter characterization and expression profiles of an antiviral gene PmAV from the shrimp (*Penaeus monodon*). *Mol. Immunol*., 44: 1516–1523

Meng, X.H., I.K. Jang, H.C. Seo and Y.R. Cho, 2010. A TaqMan real-time PCR assay for survey of white spot syndrome virus (WSSV) infections in *Litopenaeus vannamei* postlarvae and shrimp of farms in different grow-out seasons. *Aquaculture*, 310: 32–37

Miyamoto, Y., T. Saiwaki, J. Yamashita, Y. Yasuda, I. Kotera, S. Shibata, M. Shigeta, Y. Hiraoka, T. Haraguchi and Y. Yoneda, 2004. Cellular stresses induce the nuclear accumulation of importin α and cause a conventional nuclear import block. *J. Cell Biol*., 165: 617–623

Manivannan, S., S.K. Otta, I. Karunasagar and I. Karunasagar, 2002. Multiple viral infection in *Penaeus monodon* shrimp postlarvae in an Indian hatchery. *Dis. Aquat. Organ*., 48: 233–236

Natividad, K.D.T., M.V.P. Migo, J.D. Albaladejo, J.P.V. Magbanua, N. Nomura and M. Matsumura, 2006. Simultaneous PCR detection of two shrimp viruses (WSSV and MBV) in postlarvae of *Penaeus monodon* in the Philippines. *Aquaculture*, 257: 142–149

Qiao, G., D.H. Xu, Z.S. Wang, I.K. Jang, Z.T. Qi, M.M. Zhang and S.K. Kim, 2015. Comparison of immune response of Pacific white shrimp (*Litopenaeus vannamei*) after multiple and single infections with WSSV and *Vibrio anguillarum*. *Fish Shellfish Immunol*., 44: 257–264

Saitou, N. and M. Nei, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*., 4: 406–425

Song, X., C. Shi, H. Jie and L.J. Zhang, 2001. Investigation and study on carrying stat us of white spot syndrome virus (WSSV) in shrimps and other aquatic animals by dot-blot hybridization. *J. Fish Sci. Chin.*, 8: 36–40

Seewald, M.J., C. Körner, A. Wittinghofer and I.R. Vetter, 2002. RanGAP mediates GTP hydrolysis without an arginine finger. *Nature*, 415: 662–666

Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol*., 24: 1596–1599

Wang, F.X., H. Zhou, H. Ling, H.Z. Zhou, W.H. Liu, Y.M. Shao and J. Zhou, 2007. Subtype and sequence analysis of HIV-1 strains in Heilongjiang Province. *Chin. Med. J*., 120: 2006–2010

Yun, J.M., B.S. Kim, S.M. Hwang, Y.B. Kim, W.B. Choi and T.J. Choi, 2014. Artificial infection of the native Korean freshwater prawn (*Macrobrachium nipponense*) (De Haan, 1849) (Decapoda, Palaemonidae) with white spot syndrome virus (WSSV). *Crustaceana*, 87: 866–880

Yasuda, Y., Y. Miyamoto, T. Saiwaki and Y. Yoneda, 2006. Mechanism of the stress-induced collapse of the Ran distribution. *Exp. Cell Res*., 312: 512–520

Yan, D.C., S.L. Dong, J. Huang, X.M. Yu, M.Y. Feng and X.Y. Liu, 2004. White spot syndrome virus (WSSV) detected by PCR in rotifers and rotifer resting eggs from shrimp pond sediments. *Dis. Aquat. Organ*., 59: 69–73

Yan, D.C., S.Y. Feng, J. Huang and S.L. Dong, 2007. Rotifer cellular membranes bind to white spot syndrome virus (WSSV). *Aquaculture*, 273: 423–426

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